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Bacterial Expression of a Human Recombinant Monoclonal Antibody Fab Fragment Against Hepatitis B Surface Antigen

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The Fab fragment was cloned from the monoclonal cell line TAPC301-CL4, which was produced using the Epstein-Barr virus (EBV) transformation method. This cell line produces a human monoclonal antibody (CL4MAb) against the hepatitis B surface antigen (HBsAg). This MAb was shown to have hepatitis B virus (HBV) neutralizing activity in chimpanzees. The Fab fragment was produced by subjecting the heavy and light chain antibody genes of the TAPC301-CL4 cell line to reverse transcription-polymerase chain reaction, cloning the products in the plasmid vector pFab1-His2 and introducing the plasmid into bacteria. Sequence analyses of the CL4Fab fragment revealed that the light and heavy chains belong to the Vk3a and VH3 groups of the immunoglobulin (Ig) family, respectively. An enzyme-linked immunosorbent assay confirmed that specificity of the recombinant CL4Fab antibody against HBsAg was the same as that of the parental MAb. Flow cytometric analysis using PLC/PRF/5 (Alexander) cells, which express HBsAg, showed the reactivities of the CL4MAb and CL4Fab antibody were the same. These results suggest that the recombinant CL4Fab antibody produced by *Escherichia coli* using the new vector-primer system developed for human IgG Fab fragments has a very high affinity for the HBsAg and may be useful clinically. A source for generation of human MAb for human therapy with very stable and specific expression was thus produced by isolating antibodies from EBV-transformed cell lines. ***J. Med. Virol. 58:338–345, 1999.***

KEY WORDS: monoclonal antibody; anti-HBs antibody; recombinant Fab fragment; flow cytometry

INTRODUCTION

Human sera with high virus-neutralizing antibody titers are administered prophylactically against viral infection. Passive immunoglobulin (Ig) using anti-hepatitis B Ig (HBIG) effectively prevents infection of hepatitis B virus (HBV) [Prince et al., 1971; Seeff et al., 1977] in patients who undergo liver transplantation with hepatitis B surface antigen (HBsAg)-positive allografts [Muller et al., 1991] and in infants born to HBsAg-positive mothers [Seeff, 1984]. However, when HBIG is prepared from the sera of anti-HBsAg antibody-positive donors, it is difficult to ensure that the preparation is free of unknown infectious agents. Human monoclonal antibodies (MAbs) have the potential to avoid this risk. Several techniques have been developed to generate human MAbs against the HBsAg, that is, transformation of human lymphocytes by the Epstein-Barr virus (EBV) [Stricker et al., 1985; Desgranges et al., 1987; Sa'adu et al., 1992a] and fusion of human EBV-transformed lymphocytes with human [Ichimori et al., 1987] or mouse [Sa'adu et al., 1992b] myeloma cells and of human lymphocytes with mouse myeloma cells [Ichimori et al., 1985; Maeda et al., 1986] to form hetero-hybridomas. Recently, phage display technology has been utilized to generate human MAbs against the HBsAg [Zebedee et al., 1992] and this technique is a useful means of selecting antibodies. A more convenient method was established by combining an EBV-transformation method with a new primer-vector system developed for isolating human Ig Fab fragments.

An EBV-transformed cell line, TAPC301-CL4, which produces a human anti-HBsAg MAb ($\gamma 1\ \kappa$) against the common "a" determinant of the HBsAg was used. Analysis of the immune response of chimpanzees

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showed that this MAb (CL4MAb) has neutralizing activity against the HBV. Three animals received intramuscular injections of the CL4MAb Passive Hemagglutination (PHA) titer 1:128,000) 24 hr and immediately before exposure to HBV ($10^{5.5}$, chimpanzee infectious dose 50%). One of these passively immunized animals developed HBV infection, but the other two had minimal infection characterized by detectable HBsAg and transient abnormal glutamic pyruvate transaminase levels after prolonged incubation periods of 8 and 10 weeks, respectively. In contrast, one control animal that received HBV without passive immunization developed typical hepatitis B [Matsui, 1982].

In this report, the reconstruction is described of a human monoclonal Fab antibody that protects against HBV infection using a recombinant DNA technique and the TAPC301-CL4 cell line as a source of mRNA for cDNA synthesis. The genes encoding the Fab fragment of the CL4MAb were expressed in a bacterial system and the activity and specificity of the recombinant anti-HBsAg CL4Fab antibody were characterized.

MATERIALS AND METHODS

Cells

The EBV-transformed human B lymphoid cell line, TAPC301-CL4, produced a MAb (CL4) against HBsAg. This cell line was maintained in RPMI 1640 culture medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Cansera International Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin. PLC/PRF/5 (Alexander) hepatocellular carcinoma cells were obtained from the Health Science Research Resources Bank (JCRB0406; Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium culture medium (GIBCO BRL) supplemented with 10% FBS.

Cloning of Heavy and Light Chain Antibody Genes

Total cellular RNA was isolated from pelleted EBV-transformed cells using a commercial kit (RNeasy mini kit; QIAGEN). cDNAs were synthesized using random 9-mers, nucleotides, and reverse transcriptase (Takara, RNA-PCR kit, Ohtsu, Japan), and then amplified by the polymerase chain reaction (PCR) using specific primers for the heavy and light chains of human IgG. The forward primers used for VH and VK were VH3a (5′-AAGGCCCAACCGGCCATGGCCSARGTGCAGKTGGTGGAGTCTGG-3′) and VK3a (5′-CCGCTAGCGAAATTGTGWTGACGCAGTCTCC-3′), respectively. The reverse primers used for CH1 and CK were FDG1 (5′-CCGCGGCCGCTGTGTGAGTTTTGTCACAAGATTT-3′) and VKC (5′-TTGGCGCGCCACACTCTCCCCTGTTGAAGCTCTT-3′), respectively. The "touchdown" PCR protocol [Don et al., 1991] was used and a total of 11 cycles of the denaturation at 95°C for 1 min, annealing for 1 min, and elongation at 72°C for 2 min. The annealing temperature was reduced from 65°C to 55°C in increments of 1°C. The 11 touchdown cycles were followed by 25 identical cycles with an annealing temperature of 55°C. The resulting PCR product was agarose gel-purified and extracted using Qiaquick spin-columns (QIAGEN). The heavy chain Fd and light chain genes were then cloned into the *Sfi* I/*Not* I and *Nhe* I/*Asc* I sites, respectively, of the expression vector pFab1-His2 (Fig. 1). This vector was produced by modifying the pRPLS/Fab1 vector [Takekoshi et al., 1998].

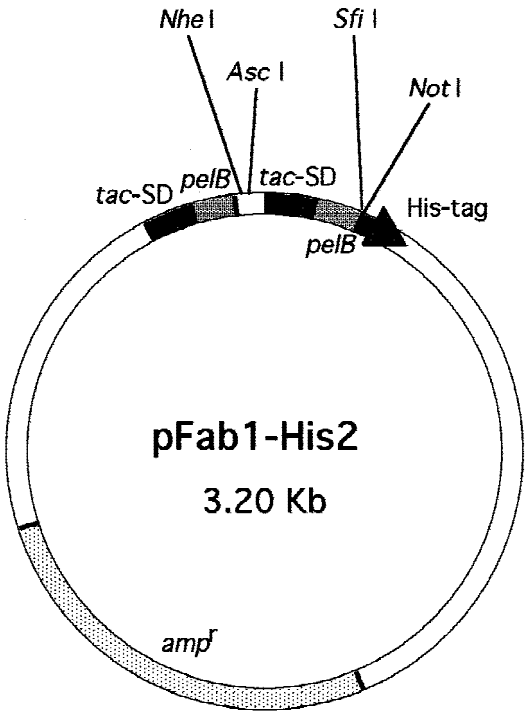

Fig. 1. Structure of the pFab1-His2 vector. The light and heavy chain genes were subcloned into the *Nhe* I-*Asc* I and *Sfi* I-*Not* I sites, respectively, of the pFab1-His2 plasmid vector. A tac promoter was used to drive both genes and the *pelB* leader sequence of *Erwinia cartovora* was used to direct Fab secretion into the periplasmic space of *Escherichia coli.* The heavy chain gene product was fused to a histidine tag. The soluble Fab fragment was produced in *E. coli.*

Expression in *Escherichia coli* and Extraction of the Soluble Fab Fragment

The ligated pFab1-His2 vector with the heavy chain Fd and light chain genes was introduced into competent *E. coli* JM109 cells (Toyobo, Osaka, Japan). After transformation, the *E. coli* cells were plated on Luria-Bertani (LB)/ampicillin (50 μg/ml) plates and then isolated bacterial colonies were incubated at 30°C in 2 ml Super Broth (SB) containing ampicillin (50 μg/ml) and $MgCl_2$ (1.5 mM). Production was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the cultures. The cells from the bacterial cultures were pelleted, resuspended in phosphate-buffered saline (PBS) containing lysozyme (1 mg/ml) and a protease inhibitor cocktail (Complete; Boehringer Mannheim), and allowed to stand for 30 min at room temperature. The cells were lysed by four cycles of freezing at −70°C and thawing at 37°C, the lysates were centrifuged at 15,000 × *g* for 30 min, and the resulting supernatant containing the Fab antibody was collected. In large-scale cul-

tures, the Fab protein fractions secreted into the periplasm of bacterial cells were collected after sonication, instead of freeze-thawing.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA plates (E.I.A/R.I.A; Costar) were coated with purified HBsAg derived from hepatoma cells (0.2 μg/well; Meiji Milk Products, Tokyo, Japan). PBS containing 1% bovine serum albumin (BSA) was added to the plates to block any nonspecific antigen-antibody reactions. CL4Fab antibodies obtained from the bacterial cultures were added to the plates and incubated for 1 hr at room temperature. A horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fab-specific antibody (Sigma) was then added and the plates were incubated for 1 hr at room temperature. Finally, tetramethylbenzidine base (GIBCO BRL) was added and the plates were incubated at room temperature and the optical density (OD) at 650 nm (OD_{650}) of the solution in each well was read. A standard reference curve was prepared by measuring OD_{650} values of serially diluted HBIG prepared from the sera of anti-HBsAg antibody-positive donors (200 IU/ml, Nihon Pharmaceutical, Tokyo, Japan) and the experimental antibody titers were determined by comparing the OD readings with the standard curve.

Nucleotide Sequence Analysis

The cloned heavy chain Fd and light chain genes in the pFab1-His2 vector that showed positive reactions with the HBsAg were recloned into sequencing vectors CV-1 and CV-2, respectively, and plasmid DNAs were prepared using a commercial kit (Qiaprep miniprep kit; QIAGEN). Cyclic sequencing in both directions of these DNAs was undertaken using a commercial kit (Thermo Sequence kit; Amersham Pharmacia Biotech), the M13 forward (5′-CACGACGTTGTAAAACGAC-3′) and reverse (5′-GGATAACAATTTCACACAGG-3′) primers and a DNA sequencer (LI-COR model 4000L, Lincoln).

Affinity Purification of IgG and Fab Antibodies

Intact CL4MAbs were purified from cell culture supernatants using an IgG purification kit (MAbTrap G; Amersham Pharmacia Biotech). For Fab fragment purification, 1 L of bacterial cell culture producing the recombinant CL4Fab antibody was lysed by sonication, the insoluble material was pelleted by centrifugation, and the supernatant was transferred to an anti-Fab antibody affinity column and eluted, as described by Harlow and Lane [1988]. The eluted Fab protein was dialyzed against PBS and concentrated by centrifugation (Centriplus 30; Amicon).

Immunofluorescence Staining and Flow Cytometric Analysis

Alexander hepatocellular carcinoma cells were cultured on plastic plates (IWAKI, Japan) and fresh culture medium was added to the semi-confluent cells 24 hr before the experiment. The cells were not fixed, but were trypsinized, washed with fresh culture medium reacted with 1 μg/ml purified CL4MAb or CL4Fab antibody for 2 hr on ice, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG-Fab (Capel) for 30 min on ice, and the cells were analyzed using a flow cytometer (CytoAce 150; JASCO, Japan).

Affinity and Kinetic Studies

The interaction between immobilized CL4Fab antibody, as the ligand, and HBsAg, as the analyte, was assayed by a method based on surface plasmon resonance using a biosensor instrument (BIACORE 2000; Amersham Pharmacia Biotech). The nitrilotriacetate (NTA) sensor chip was activated with a solution of 500 μM of $NiCl_2$ in running buffer, which comprised 10 mM HEPES, 150 mM NaCl, and 50 μM ethylenediamine tetraacetic acid (EDTA; pH 7.4), at a flow rate of 20 μl/min for 1 min. Unpurified lysate containing the CL4Fab antibody (diluted 1:100 with running buffer) was immobilized on the nickel-activated NTA sensor chips at a flow rate of 2 μl/min for 10 min. Solutions (250 μl) of HBsAg, at four different concentrations (32, 63, 125, and 250 μg/ml in running buffer) were each injected onto an antibody-coated sensor chip at a flow rate of 20 μl/min and then disassociated for 5 min. When the interaction analysis was complete, the sensor chips were returned to their original state with a solution comprising 350 μM EDTA, 10 mM HEPES, and 150 mM NaCl at a flow rate of 10 μl/min for 2 min, followed by washing with the running buffer at a flow rate of 20 μl/min for 10 min. Changes in the surface plasmon resonance were monitored by the BIACORE 2000 biosensor system and the kinetic constants k_{on} (association rate constant), k_{off} (dissociation rate constant) and K_d (dissociation constant, k_{off}/k_{on}) were calculated by nonlinear fitting performed by the evaluation program.

RESULTS

cDNA Cloning and CL4Fab Fragment Expression

To obtain cDNA clones for the reconstructed CL4Fab fragment, total cellular RNA was isolated from human anti-HBsAg antibody-producing cells, both the Fd region and light chain genes were reverse-transcribed, PCR-amplified and cloned into the pFab1-His2 vector (Fig. 1); expression of both genes was controlled by the *E. coli tac* promoter. As indicated in Figure 1, a histidine tag was added to the heavy chain produced by this vector and the light chain was expressed independently. A *pelB* signal sequence of *Erwinia carotovora* was fused to both genes to direct protein secretion into the periplasmic space of the bacterial host. To identify specific bacterial clones expressing the Fab antibody of interest, the reactivity of each Fab with the HBsAg was screened by ELISA. All the colonies expressed an antibody directed against HBsAg (data not shown). One of the clones, named CL4Fab, was selected and examined further.

Heavy chain

1:EVQLLESGGGVVQPGRSLRLSCIASGFTFS SYPMT WVRQAPGKGLEWVA SISYDGSYKYK
FR1 CDR1 FR2 CDR2

61:VDSMKG RLTISRDNSKNTLYLEMNSLTAEDTAVYYCAR TAFFNAYDF WGQGTLVTVSS
FR3 CDR3 FR4

Light chain

1:EIVMTQSPATLSVSPGERATLSC RASQSVRSNLA WYQQKPGQAPRLLIY AASTRA TGIPA
FR1 CDR-1 FR2 CDR-2

61:RFSGSGSGTEFTLTISSLQSEDFAVYYC QQYNEWFRT FGQGTKVEIKR
FR3 CDR3 FR4

Fig. 2. Deduced protein sequences of the heavy and light chain V-genes of the CL4Fab fragment. Abbreviations: CDR, complementarity-determining region; FR, framework region. The numbers before the amino acid sequences represent the order of appearance of the sequences.

DNA Sequence Analysis

The amino acid sequences of the heavy and light chain variable regions, deduced from the base pair sequences of the CL4Fab clone DNA, are shown in Figure 2. These sequences were compared with those in nonredundant DNA databases (DDBJ, GenBank, EMBL) using the MPsrch program (http://disc.dna.affrc.go.jp/). The findings indicated that the sequences of CL4Fab belonged to the human Ig family γ1,VH3:k3a, but were not identical to any other sequences in the DNA databases. This heavy chain Fd region was 89.3% homologous with the heavy chain of human anti-HBsAg Ig (unpublished data, accession number U92452). The CL4Fab light and heavy chain variable regions were also compared with the sequences in the Kabat database (http://immuno.bme.nwu.edu/). The light chain and heavy chain framework regions and complementarity-determining regions 1 and 2 were almost identical to those of the human κ germ-line Humkv328 [Liu et al., 1989] and human VH CLONE38 [Welschof et al., 1995] genes, respectively. The nucleotide sequence data for the recombinant Fd domain and recombinant κ-chain reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB012909 and AB012910, respectively.

Purification and Characterization of the Recombinant Fab Antibody

To characterize the recombinant CL4Fab antibody further, Ig production by bacterial cultures containing the recombinant plasmid was induced by adding 50 μg/ml IPTG, and the CL4Fab antibody was collected and purified by affinity chromatography. The IgG MAb secreted by the lymphoid cell line TAPC301-CL4 into the culture medium was also purified by affinity chromatography. The purities of the CL4MAb and the CL4Fab antibody were assessed by 12.5% sodium do-

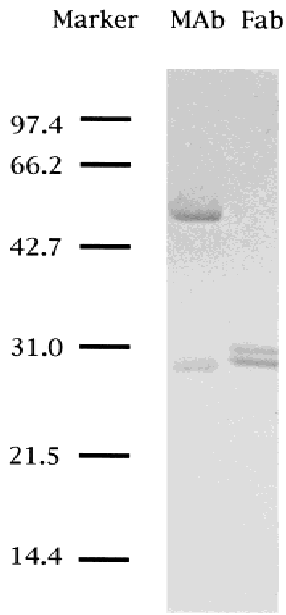

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the CL4MAb and CL4Fab fragment molecules. Affinity-purified CL4MAb and CL4Fab antibody were subjected to SDS-PAGE (12.5%).

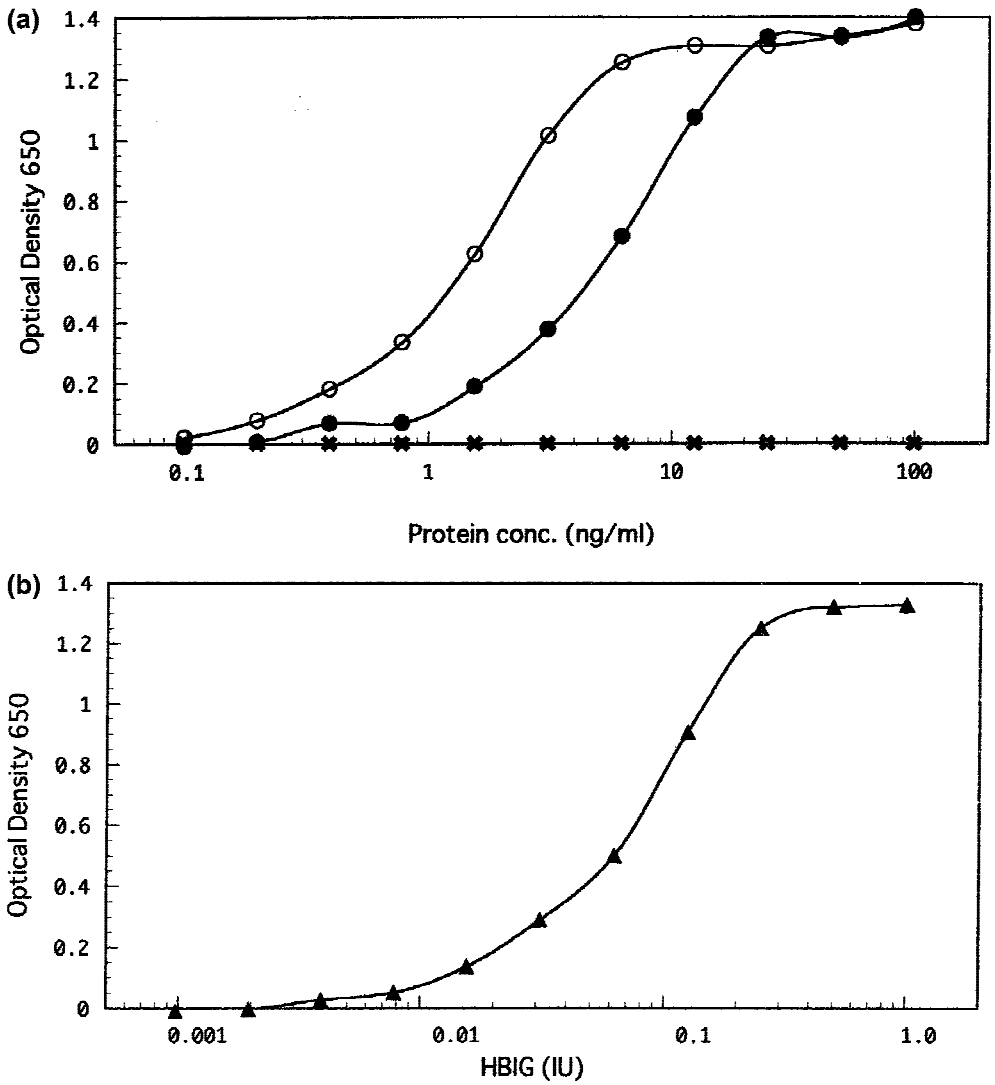

Fig. 4. Quantification of anti-hepatitis B surface antigen (anti-HBsAg) antibodies by enzyme-linked immunosorbent assay (ELISA). **(a)** Serially diluted solutions of CL4MAb (●) and CL4Fab (○) were incubated in microtiter plates precoated with 200 ng/well HBsAg. The monoclonal antibody Fab13-3 (X) was used as a negative control. **(b)** Hepatitis B immunoglobulin (HBIG) (▲), which is used for passive immunoprophylaxis against hepatitis B virus, was used as a positive control. Assays of CL4MAb, CL4Fab, Fab13-3, and HBIG were carried out on the same plate simultaneously.

decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, as described by Laemmli [1970]. The purified MAb yielded two bands with molecular masses of approximately 55 and 28 k (Fig. 3, MAb). The approximate molecular masses of the Fd and light chains were 34 and 30 k, respectively (Fig. 3, Fab). The light chain gene product was slightly heavier than the light chain of the CL4MAb, because we had added 9 amino acids to the 5′- and 3′-ends to create unique restriction enzyme sites for *Nhe* I and *Asc* I (Fig. 1), which were used for cloning of the PCR-amplified light chain gene. The apparent molecular masses of the CL4Fab antibody and light and heavy chains were almost 5 k heavier than the masses predicted from their amino acid sequences. The CL4MAb light chain was also 5 k heavier than expected. The reason why these light and heavy chains molecules were larger than predicted is unknown, although Western blotting confirmed that both the light and heavy chains of the CL4MAb and the CL4Fab antibody were specific for human Ig (data not shown).

Quantitative Analysis of the Recombinant Fab

The antigen binding activities of the purified CL4Fab proteins were determined by ELISA. As shown in Figure 4, the activities of the purified anti-HBs antibodies were quantified by comparing their OD_{650} values with those of serially diluted solutions of a standard HBIG preparation (Nihon Pharmaceutical, Japan), which is currently used, at a dose of 1,000–2,000 international units (IU)/time, for passive immunoprophylaxis against HBV. The molecular mass of the CL4Fab antibody was one-third that of the CL4MAb, and these antibodies had one and two antigen-binding

sites, respectively. Although it is difficult to directly the activities of the MAb and Fab antibody compare, taking the differences in their sizes and the numbers of antigen-binding sites into consideration, the amounts of the antibodies produced, estimated from their absorbances of about 0.6–0.7 at OD_{650}, the values at which 50% maximal binding was observed (shown in Fig. 4a, b), were 50 ng CL4MAb, 20 ng CL4Fab antibody, and 1 IU HBIG. A monoclonal antibody Fab13-3, against human cytomegalovirus (HCMV) [Takekoshi et al., 1998] was used as the negative control and it showed no reactivity in this ELISA.

Immunofluorescence Staining and Flow Cytometric Analysis

Alexander cells, human primary liver cancer cells that synthesize HBsAg, were subjected to indirect immunofluorescence staining with the CL4MAb and CL4Fab antibody. The surfaces of live Alexander cells showed patchy staining patterns when stained with those two antibodies (data not shown). As shown in Figure 5, the fluorescence intensities of cells that reacted with the CL4MAb or CL4Fab antibody against HBsAg were higher than that of control cells reacted with the monoclonal antibody Fab13-3 against HCMV. Therefore, both these antibodies reacted with HBsAg synthesized by Alexander cells.

Affinity and Kinetic Studies

The binding affinity of the CL4Fab antibody was investigated by carrying out surface plasmon resonance analysis. The CL4Fab antibody produced possessed a histidine tag, which enabled analysis of the interaction between the CL4Fab antibody and the HBsAg based on surface plasmon resonance to be performed using an NTA sensor chip. The overlay plot of the interaction profiles of four different concentrations of the HBsAg is shown in Figure 6. The association rate constant (k_{on}) for the binding of the HBsAg to immobilized CL4Fab antibody was 1.9×10^3 M^{-1} s^{-1}, and the dissociation rate constant (k_{off}) was 1.2×10^{-4} M^{-1} s^{-1}. Therefore, the dissociation constant (K_d), which is defined as koff/kon, was estimated to be 6.310^{-8} M^{-1}. The monoclonal antibody Fab13-3 against HCMV showed no detectable specific binding with the HBsAg at any concentration tested (32, 63, 125, and 250 μg/ml in running buffer; Fig. 6, e, f, g, h).

DISCUSSION

A human Fab gene fragment was cloned using EBV-transformed cells that produced HBV-neutralizing MAbs and expressed the recombinant Fab in *E. coli.* Several anti-HBs MAbs from human sources have been developed. Several types of human antibody been reported to be produced from human peripheral blood mononuclear cells transformed by EBV [Stricker et al., 1985; Desgranges et al., 1987; Ichimori et al., 1987; Sa'adu et al., 1992a]. It is important that antibodies for clinical use are free from EBV contamination and one approach to overcome the problem of such contamina-

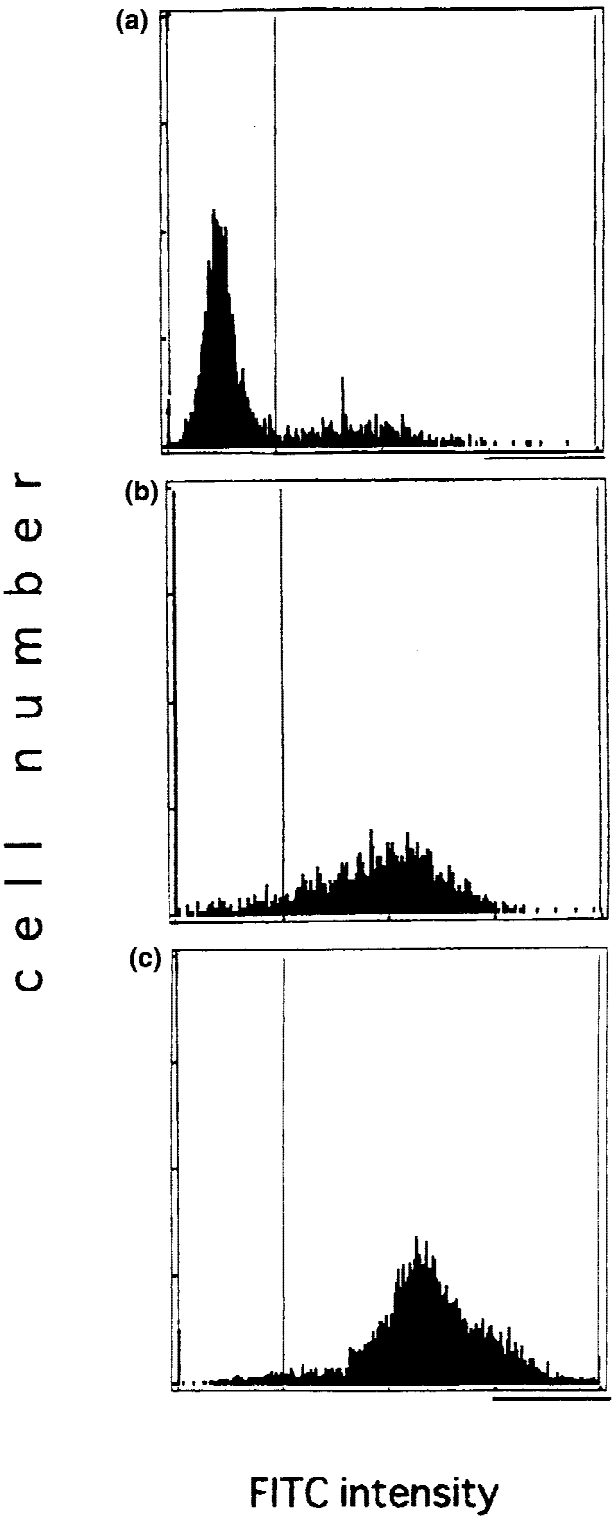

Fig. 5. Flow cytometric analysis of the binding activities of the CL4MAb and CL4Fab antibody with hepatitis B surface antigen (HBsAg). Trypsinized Alexander cells were subjected to indirect fluorescence labeling and the cells labeled with each antibody were analyzed using a flow cytometer. The number of cells and immunofluorescence intensity are shown on the vertical and horizontal axis, respectively. The monoclonal antibody Fab13-3 was used as a negative control. **a:** Negative control; **b:** CL4MAb; **c:** CL4Fab.

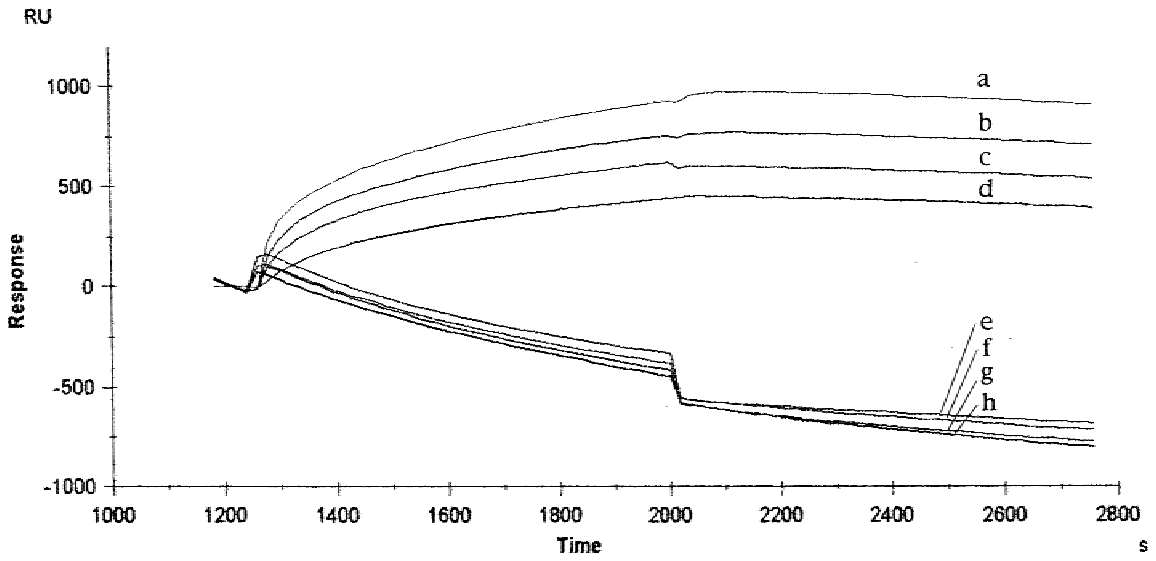

Fig. 6. Surface plasmon resonance analysis. Hepatitis B surface antigen (HBsAg) (250 μl) at four different concentrations (32, 63, 125, and 250 μg/ml in running buffer) was injected onto CL4Fab antibody-coated sensor chips and the surface plasmon resonance was monitored using a biosensor system. HBsAg (μg/ml): a and e, 250; b and f, 125; c and g, 63; d and h, 32. The monoclonal antibody Fab13-3 was used as a negative control. a–d, CL4Fab; e–h, Fab13-3.

tion is to clone antibody genes and generate their Fab fragments in *E. coli.* The reactivities in vitro and in vivo of our human recombinant CL4Fab antibody produced in *E. coli* were identical with those of the parental CL4MAb (Figs. 4 and 5, respectively). According to our ELISA results, the CL4Fab antibody titer was very high; 20 ng CL4Fab was equivalent to 1 IU HBIG (Fig. 4). For clinical applications, antibodies with high affinities and high specificities are required. In this respect, our recombinant CL4Fab antibody appears to have high enough affinity and specificity for the HBsAg and it is free from EBV contamination. However, it did not recognize denatured HBsAg (data not shown). Most anti-HBs MAbs that bind to the "a" group determinant of the HBsAg have been demonstrated to recognize conformational rather than linear epitopes [Sa'adu et al., 1991]. To date, in our laboratory, we can prepare at least 1 mg affinity-purified Fab, which corresponds to 50,000 IU HBIG, from 100 ml culture medium. Usually, 1,000–2,000 IU/time are needed for passive immunoprophylaxis.

E. coli is the most popular expression host for Fab fragments because bacterial expression of antibody fragments is cheaper, faster, and easier than expression by mammalian cells. In future, human anti-HBsAg Fabs produced by bacteria may be used more often than the currently available HBIG. Combinatorial antibody libraries generated from human sources have been used to isolate a human antibody Fab fragment against HBsAg [Zebedee et al., 1992], but whether this Fab antibody prevented HBV infection was not clear. This technique also utilizes the *E. coli* expression system.

A cocktail of MAbs against HBsAg was reported to induce higher titer responses than each MAb alone [Harada et al., 1989; Heijtink et al., 1995] and the cocktail neutralized HBV infection of a chimpanzee [Harada et al., 1989]. A MAb cocktail may lead to reduced use of HBIG. The HBsAg is composed of three antigens, preS1, preS2, and S, all three of which are targets of neutralizing antibodies [Neurath et al., 1986; Quiroga et al., 1987]. Therefore, MAbs against these three antigens in a cocktail may act synergistically.

Further work is in progress to produce a cocktail composed of Fab antibodies that recognizes anti-preS1, preS2, S, and different HBs epitopes.

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